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Veterinary Parasitology 113 (2003) 211–216
veterinary parasitology
www.elsevier.com/locate/vetpar

A rapid MTT colorimetric assay to assess the proliferative index of two Indian strains of *Theileria annulata*

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Received 9 August 2002; received in revised form 14 January 2003; accepted 25 January 2003

Abstract

A study was undertaken to compare the proliferative index of macroschizont-infected lymphoblastoid cells of two Indian strains [Izatnagar (IZT) and Parbhani (PBN)] of *Theileria annulata* by an in vitro MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], colorimetric assay. Culture conditions were standardized to define the optimal cell concentration in 96-well microculture plates to yield nearly 100% living cells for measurement of the metabolized formazan activity. A cell concentration of 1.5×10^5 cells/ml was found to be optimal for effective discrimination of the parasite strains. On the basis of conversion of MTT by the actively proliferating lymphoblastoid cells, the PBN strain of *T. annulata* stimulated a 2.5-fold increase in formazan activity in comparison to the IZT strain. The in vitro MTT assay was found to be a simple and convenient method for assessing the cell activation rate and growth, obviating the need for radioactive material for the assay. The results of the proliferation assay are discussed in relation to previously documented information on the biological characteristics of this important pathogen of cattle.

Keywords: *Theileria annulata*; Replication index; MTT proliferation assay

1. Introduction

Theileria annulata causes bovine tropical theileriosis in cattle across a wide zone of Africa, Europe and Asia. The distribution of the disease is dependent on the presence of susceptible animals and the vector ticks (*Hyalomma* spp.). In India, *T. annulata* is the

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doi:10.1016/S0304-4017(03)00062-1

predominant species, cyclically transmitted by *Hyalomma anatolicum anatolicum*, while isolated reports of *T. orientalis* are also on record (Shastri et al., 1985). The control of the disease is mainly achieved by chemotherapy and vaccination of the susceptible stock by attenuated schizont vaccine combined with vector control measures. Although, the antigenic diversity in *T. annulata* is not well known, there are occasional reports of vaccination failures in India (Subramanian et al., 1988). Existence of strains of *T. annulata* of varying virulence has been reported from Algeria, Israel, Iran and Iraq (Sergent et al., 1945; Wilde, 1967; Pipano et al., 1974). The commonly recognized *T. annulata* strains, named after their place of origin in India are Izatnagar, Hisar, Ludhiana, Anand, Parbhani and Chennai (formerly Madras). Information on the extent of variation in virulence among these *T. annulata* strains, is scanty. However, parasite stocks are known to exhibit different levels of virulence and cross-protection.

Deshpande (1989) made some early observations on the virulence and the cross-protection between two Indian strains derived from Parbhani (PBN) and Izatnagar (IZT). Bansal and Ray (1994) made critical observations on the antigenic diversity between these two strains. They observed that young bovine calves spontaneously recovered from naturally acquired *T. annulata* infection were protected against the homologous challenge with IZT strain, but were susceptible to challenge with PBN strain.

Saravanan et al. (2003) on the basis of comparative clinico-parasitological observations of these strains regarded PBN strain as a distinct strain, with higher virulence in comparison to the Izatnagar strain. The severity of theileriosis usually depends on susceptibility of host, quantum of infection delivered by vector ticks and parasite virulence. The latter is often assessed by in vivo replication rate of the parasites and/or antigenic differences among the strains (Robinson, 1982). However, there is no marker system to discriminate the replication rate of the *T. annulata* parasites in vitro for correlation with virulence of the organisms. The MTT tetrazolium salt [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], colorimetric assay has been previously described (Mosmann, 1983) to measure cytotoxicity and cell proliferation and as an index of measurement of cell activation (Gerlier and Thomasset, 1986) both qualitatively and quantitatively. We report in this study the suitability of MTT colorimetric assay as an in vitro correlate of the replication rate of metabolically active *T. annulata* macroschizont-infected lymphoblastoid cells.

2. Materials and methods

2.1. Parasite cell lines

IZT and PBN isolates of *T. annulata* macroschizont-infected bovine lymphoblastoid cells were used in the experiment. Cultures were established using cell lines available in the Theileria Laboratory of the Division of Parasitology. Cultures were set up using cryopreserved stabilates, containing 2×10^5 infected bovine lymphoblasts derived from IZT and PBN strains of *T. annulata* in RPMI-1640 medium (Sigma) supplemented with 10% neonatal calf serum in 25 cm^2 culture flasks. Cultures were incubated at 37 °C in a BOD incubator and periodically examined using an inverted microscope (Olympus, Japan).

The cultures were maintained by continuous passage. The schizont-infected lymphoblastoid (SIL) cells, in the active growth phase in cell culture, were used for MTT colorimetric assay.

2.2. MTT colorimetric assay

The rate of replication of each of the SIL cell lines was determined by lymphoproliferative assay using MTT dye [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], according to the method described by Mosmann (1983), Bounous et al. (1992) and Carmichael et al. (1987) with slight modifications. The assay was carried out as follows. SILs of both strains were harvested from a 25 cm^2 flask, pelleted by centrifugation and resuspended in approximately 2 ml of fresh growth medium. The viable cells were counted by 0.4% trypan blue dye exclusion test and the cell count was adjusted to 2×10^6 cells/ml. A pilot experiment was performed using a cell concentration of 2×10^6 cells/ml (100 μl per well) in quadruplicate wells in a 96-well microculture plate (Nunc, Denmark). The culture plate was incubated at 37 °C in a CO_2 incubator (Queue, USA) for 24 h. Control wells for both parasite strains were prepared by harvesting SILs from a fresh culture flask and plating cells at a concentration of 2×10^6 cells/ml (100 μl per well), in quadruplicate wells, as described earlier. The cultures were examined by interference light microscopy (Olympus, Japan) to assess the status of the cells. The medium was than removed from all the wells after centrifugation of the plate at 1000 g for 20 min. About 20 μl of MTT dye (5 mg/ml of phosphate buffered saline (pH 7.2) stock, Sigma) was added to all the wells and the plate was incubated at 37 °C in a CO_2 incubator. After 4 h, 150 μl of dimethylsulfoxide (DMSO, Sigma) was added to all the wells to dissolve the formazan crystals and the optical density (OD) was measured at a test wave length of 490 nm and reference wavelength of 650 nm on UVmax kinetic microplate reader (Molecular Devices). The OD of the test wells after 24 h of culture were too low for any objective comparison with the OD of control wells assayed immediately after plating. Accordingly, experiments were conducted with various cell concentrations ranging from 1.5×10^2 to 1.5×10^6 cells/ml (100 μl per well) for each dilution in quadruplicate wells to determine the optimal cell concentration for comparison of the in vitro growth of cells. Replication index (RI) was calculated from the formula RI = OD of SILs at 24 h/OD of SILs at 0 h.

3. Results

Microscopic examination of the bovine lymphoblasts infected with either PBN or IZT strains of *T. annulata* revealed characteristic growth pattern after 24 h depending on initial seeding density [1.5×10^2 to 1.5×10^6 cells/ml (100 μl per well)]. The first requirement for standardizing the assay to determine the optimal seeding concentration as a function of its initial cell number plated for consistent measurable colour production and comparison. When the cultures were plated with a concentration of 1.5×10^2 to 1.5×10^4 cells/ml, no consistent colour production could be visualized. When the cultures were seeded with a concentration of 1.5×10^6 cells/ml, no appreciable difference in the cell populations was seen. A cell concentration of 1.5×10^5 cells/ml was however found to be optimal for this purpose.

Table 1
Macroschizont-infected lymphoblastoid cell proliferation (RI) by *Theileria annulata* strains after 24 h in vitro incubation (mean ± S.E.; $n = 4$)

Cell concentration (cells/ml)	PBN	IZT
1.5×10^6	1.147 ± 0.078	1.003 ± 0.088
1.5×10^5	5.002 ± 0.546^a	2.007 ± 0.355^b
1.5×10^4	0.897 ± 0.191	0.508 ± 0.024
1.5×10^3	0.894 ± 0.163	0.610 ± 0.159
1.5×10^2	0.673 ± 0.252	0.641 ± 0.092

Mean values with superscripts (a and b) differ significantly ($P < 0.05$).

The data of the RI of the PBN and IZT strains of *T. annulata* are presented in Table 1.

There was a significant difference in the OD values between the test and control wells of the strains at a concentration of 1.5×10^5 cells/ml in comparison to other test groups. The increase in absorbance values between the test wells of PBN and IZT strains at 1.5×10^6 cells/ml concentration was non-significant. In the same group, the absorbance values of the test wells of IZT strain was found to be less than the absorbance values of control wells. There was also no significant difference in the absorbance values between the test and control wells of the same strain (intra) or between the test wells of different (inter) strains in the wells with lower dilution (1.5×10^4, 1.5×10^3 and 1.5×10^2 cells/ml).

4. Discussion

The MTT assay is based on the capacity of the mitochondrial enzyme, succinate-dehydrogenase of viable cells to transform the MTT tetrazolium salt into a blue coloured product, MTT formazan and is proportional to the number of living cells present.

The assay is used primarily in mammalian cell studies as a measure of cell activation (Gerlier and Thomasset, 1986), cell growth and survival (Ficken et al., 1991; Mosmann, 1983), the bactericidal activity of macrophages (Peck, 1985); and to determine the chemosensitivity of cell lines (Riley et al., 1988) and viability of fungi (Levitz and Diamond, 1985).

The cell activation in response to a exogenous mitogen at different concentrations is read after 72 h of culture by formazan production with the dye. Adaptation of MTT assay for evaluation of the RI between the parasite strains in question, needed accurate determination of optimal cell concentration of the test, SILs under the culture conditions described. As the doubling time of SILs is 18–24 h, an initial cell concentration of 2×10^6 cells/ml was found to result in overgrowth of cells. Despite higher cell number, the number of metabolically active cells as measured by conversion of MTT was low in comparison to the freshly seeded SILs (control). For correct interpretation of the performance of the MTT assay judged by conversion of MTT dye with the actively proliferating lymphoblastoid cells of the test and controls, it required establishing optimal cell concentration for effective discrimination of the test and control SILs.

Following the assay standardization, optimal concentration of cells to microculture plate was found to be 1.5×10^5, as cell concentrations of 1.5×10^4 to 1.5×10^2 cells/ml dispensed

in 100 μl total volume were found to be either dead or low in measurable formazan activity. However, cells in concentration of 1.5×10^6 cells/ml of the two strains showed varying results. Given the fact that cells distributed in various wells at various concentrations were all from a common source, it is likely that wells receiving cell density of $<1.5 \times 10^5$, i.e. 1.5×10^4 to 1.5×10^2 cells/ml set to 100 μl total culture medium were either inadequate or failed to meet the optimal cell density for growth. On the other hand, it was interesting to observe that wells receiving IZT strain of *T. annulata* at 1.5×10^6 cells/ml may have caused an overcrowded condition resulting in mostly dead cells 24 h after incubation and consequently a lower formazan activity in comparison to zero hour cultures. At the same time, the wells receiving an identical number of lymphoblastoid cells infected with schizonts of PBN strain showed comparatively higher formazan activity (than IZT strain seeded wells). It is therefore, likely that the PBN strain of *T. annulata* generated more daughter cells and higher degree of formazan activity due to higher parasite in vitro replication.

The results of the MTT assay suggest 2.5-fold increase in formazan activity in PBN strain of *T. annulata* in comparison to IZT strain, which is a reflection of higher proliferation or growth potential of the former. The higher proliferation index in turn may explain the inherent virulence of the isolate. A positive correlation between higher virulence with faster growing (in vitro) clones of *T. annulata* has been recently demonstrated (Taylor et al., 2002). The relationship between these two traits have also been elucidated for clones of *Babesia bovis* in cattle (Nevils et al., 2000), *Plasmodium chabaudi* in mice (Mackinnon and Read, 1999) and *Schistosoma mansoni* in mice (Davies et al., 2001). Further, assessment of parasite growth rates in culture was also successfully used to predict the virulence of *B. bovis* in cattle (Nevils et al., 2000). It is also hypothesized that growth rate and virulence characteristics of *Theileria* parasites must be a product of both parasite and host factors (Taylor et al., 2002). However, the growth rates of these parasites in vivo are hard to measure due to their tissue tropisms. From the present study, it may be concluded that *T. annulata* strains show varying degree of replication in vitro and the replication index studied by the MTT assay described here may serve as an in vitro correlate of higher virulence trait of the PBN strain of *T. annulata*. These results correlate with the previously documented clinico-parasitological studies of the parasite and reaffirm that the parasite growth rates may play a positive role in determining the virulence to the host.

Acknowledgements

The authors are grateful to Dr. Mohini Saini, Scientist, Division of Biochemistry and Food Sciences and Head, Division of Parasitology and Director, Indian Veterinary Research Institute, Izatnagar, for providing necessary facilities. Dr. B.C. Saravanan is grateful to ICAR, New Delhi, for awarding Junior Research Fellowship during this programme.

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